ON PHYSIOLOGICALLY BALANCED SOLUTIONS FOR BACTERIA (B. SUBTILIS)

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In a former paper[1] I have shown that the laws governing the toxic and anti-toxic effects of salts, which LOEB and OSTERHOUT have found to hold so universally for animals and plants respectively, hold also for bacteria if we may consider *B. subtilis* as representative of this group. I shall now attempt to show that as regards complete physiologically balanced solutions, the same general relation holds for bacteria as for animals and plants.

In his experiments on a marine fish (Fundulus) LOEB was able to show that the views of HERBST regarding the absolute necessity for each of the constituents of sea water for the complete development of the egg were erroneous, since Fundulus, which would not develop in a pure NaCl solution of the same osmotic pressure as sea water, would live indefinitely in distilled water, and therefore the salts contained in sea water were necessary, not for nutrient purposes, but for the purpose of overcoming toxic effects of other salts, or briefly for balancing purposes. From this and other experiments in which he was able to overcome the toxicity of NaCl solutions by the addition of small amounts of salts like $ZnSO_4$, $BaCl_2$, and $Pb(CH_3COO)_2$, which are themselves exceedingly toxic, LOEB concluded that physiologically balanced solutions, in which one constituent overcomes the toxic effect of one or more others, are essential for the proper development of animals, and further that the blood and sea water may be considered such balanced solutions in nature.

In similar experiments on plants OSTERHOUT[2] showed that LOEB'S conception of "physiologically balanced solutions" holds good also for that class of organisms. In many series of tests on a great variety of marine and freshwater plants that investigator proved that the combination of NaCl, KCl, and $CaCl_2$ in the proportions in which they exist in sea water is just as uniformly beneficial for plants as for animals.

[1] BOT. GAZETTE 48:105–125. 1909.

[2] Univ. Calif. Publ. Bot. 2:no. 10. 1906.

From the interesting results just cited, it was deemed important to ascertain if, contrary to the opinion of LOEW and ASO,[3] physiologically balanced solutions are necessary for bacteria as they have been found to be for plants and animals, and if the behavior of bacteria toward completely balanced solutions is in full accord with that of the other classes of organisms. Accordingly, seven series of experiments were carried out, in which were employed upward of thirty solutions of different composition. In order to have the results strictly comparable, the average of several determinations in each case is inserted in the tables. It may be said that the duplicates showed very close agreement for ammonification cultures. The technique employed in these experiments was in general the same as that described in my former experiments above cited. The cultures were incubated in Erlenmeyer flasks of 250^{cc} capacity for 2.5 days, at the end of which time the ammonia was determined. The solutions in each flask consisted of the constituents described under each in the tables, and, in order to provide uniform surfaces of aeration, were reduced to a bulk of 100^{cc} in each flask after the salts were combined in the proportions given.

SERIES I

The object here was to compare pure solutions of NaCl and KCl and combinations of them with $MgCl_2$ and $CaCl_2$ respectively (which combinations have been shown to be beneficial in the experiments with binary solutions) with a blank solution of peptone in distilled water and one of peptone in tap water. The results are given in table I.

In this series, as well as in former experiments, the salt mixtures which contained NaCl as the predominant salt always seemed to give better results, and it was decided therefore in the following series to make extensive use of the NaCl as a basis for all artificial solutions. We see from the table that the best results obtained with the salt solutions was in solution 3, as was the case in the experiments with binary solutions, but it will also be noticed that the blank peptone solutions in both tap and distilled water gave very much higher results than even solution 3. The depressing effect seen in the tap

[3] Bull. Coll. Agric. Imp. Univ. Tokyo 7:355. 1907.

water peptone solution when compared with the distilled water culture is possibly due to the fact that small quantities of toxic salts

TABLE I

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS
PEPTONE CONTENT 0.91 PER CENT.

Number	Culture solution	N as NH_3 formed in cultures, in mg
1	100 NaCl	20.10
2	100 KCl	15.38
3	{ 100 NaCl; 10 $MgCl_2$ }	23.39
4	{ 100 KCl; 5 $CaCl_2$ }	17.90
5	Peptone in distilled water	42.51
6	Peptone in tap water	31.90

are dissolved from the metal pipes by the water and thus have an unfavorable effect on the growth and development of *B. subtilis.*

SERIES II

The next step taken was to ascertain if salt mixtures containing three salts instead of two provide better conditions for ammonification

TABLE II

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS
PEPTONE CONTENT 0.91 PER CENT.

Number	Culture solutions	N as NH_3 formed in cultures, in mg
1	{ 100 NaCl; 10 $MgCl_2$; 10 KCl }	20.63
2	{ 100 NaCl; 10 $MgCl_2$; 10 $CaCl_2$ }	18.91
3	{ 100 NaCl; 10 $MgCl_2$; 5 $CaCl_2$ }	25.88
4	{ 100 NaCl; 10 $MgCl_2$; 25 KCl }	24.90
5	{ 100 NaCl; 10 $MgCl_2$; 50 KCl }	22.10
6	{ 100 NaCl; 10 $MgCl_2$ }	23.39

by *B. subtilis* in peptone solutions. The solutions were prepared as shown in table II, and the ammonia determined with results as given.

Comparing the first five solutions with solution 6, we find that we have not yet arrived at what might be termed a balanced solution. Some salt mixtures of three salts do not give as good results as solution 6, while solutions 3 and 4, which appear to be better than the former, are not sufficiently near the peptone water cultures (see table I) to allow them to stand without further experiments as the optimum solutions. None the less, it is plain that solution 3 is approaching the balanced solution.

SERIES III

From the results just given it was thought that the addition of another salt to the mixtures of three salts might tend to make them

TABLE III

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS PEPTONE CONTENT 0.01 PER CENT.

Number	Culture solution	N as NH_3 formed in cultures, in mg
1	100 NaCl, 10 $MgCl_2$, 10 $CaCl_2$, 10 KCl	19.37
2	100 NaCl, 10 $MgCl_2$, 5 $CaCl_2$, 10 KCl	18.91
3	100 NaCl, 5 $CaCl_2$, 10 $MgCl_2$, 25 KCl	21.75
4	100 NaCl, 10 $CaCl_2$, 10 $MgCl_2$, 25 KCl	22.87
5	100 NaCl, 10 $MgCl_2$, 5 $CaCl_2$	25.88
6	100 NaCl, 10 $MgCl_2$	23.30

approach still closer than did solution 3 of the last series the optimum solution. There were prepared, therefore, solutions containing

various combinations of four salts each, and for comparison with them solution 3 of table II containing three salts and solution 6 of table II containing only two salts.

From table III it is plain that the various combinations of four salts, though allowing a fairly good production of ammonia, are not as favorable to *B. subtilis* as either 5 or 6, which contain respectively only three and two salts. It appears evident that the combinations of four salts there employed have not the favorable composition of a balanced solution, and since it could not easily be ascertained as to what was the depressing agent in these solutions, it was decided to run another series with different combinations of four salts each, to see if any other combination of four salts can more nearly be made to approach the balanced solution.

SERIES IV

Since calcium proved to be so toxic a salt to *B. subtilis*,[4] it was thought that an improvement could be made in the combinations of four salts by using smaller amounts of calcium than were employed in the last series, and solutions of four salts each (shown in table IV)

TABLE IV

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS
PEPTONE CONTENT 0.91 PER CENT.

Number	Culture solutions	N as NH_3 formed in cultures, in mg
1	100 NaCl, 25 KCl, 10 $MgCl_2$, 2 $CaCl_2$	20.70
2	100 NaCl, 25 KCl, 10 $MgCl_2$, 1 $CaCl_2$	21.12
3	100 NaCl, 10 KCl, 10 $MgCl_2$, 2 $CaCl_2$	28.42
4	100 NaCl, 10 $MgCl_2$, 5 $CaCl_2$	25.88
5	Peptone in distilled water	42.51
6	Peptone in tap water	31.90

[4] LIPMAN, CHAS. B., BOT. GAZETTE **48**: 105–125. 1909.

were arranged and compared with solution 5 of the last series containing three salts and with a solution of peptone in distilled water and one in tap water.

The large proportion of the potassium salt interfered with the beneficial action of the calcium in solutions 1 and 2, as can be seen by comparing them with solution 3, which differed from the others only in having less potassium and in consequence seems to approach quite closely the peptone solution in tap water, and also is far better than solution 4, which until now showed up as the most favorable solution of all the artificial salt mixtures employed. Solution 5 as before shows the optimum conditions, since there is neither high osmotic pressure nor any toxicity in it to interfere with the ammonification by *B. subtilis*.

SERIES V

Since in the foregoing series we have been gradually approaching a balanced solution in some of the salt mixtures, it is appropriate to compare them now with sea water, both in its original and diluted forms, as well as with RINGER'S solution (which consists of 100 parts NaCl, plus two parts KCl, plus two parts $CaCl_2$), and with a solution

TABLE V

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS, EXCEPT AS EXPLAINED ABOVE

Number	Culture solution	N as NH_3 formed in cultures, in mg
1	100 NaCl, 10 $MgCl_2$, 5 $CaCl_2$	25.88
2	100 NaCl, 10 KCl, 10 $MgCl_2$, 2 $CaCl_2$	28.42
3	RINGER'S solution	19.74
4	Natural sea water	10.72
5	Dilute sea water	31.35
6	Artificial sea water (VANT' HOFF'S solution)	31.41

made up so as to constitute an artificial sea water (VAN'T HOFF'S solution). The natural sea water was obtained from Pacific Grove, California, and by titration for chlorids was found to have a con-

centration of 0.53 *m with an alkaline reaction.* The dilute sea water was made by adding distilled water to the concentrated sea water until a concentration of 0.35 *m* (or that used in all the other solutions) was obtained. The artificial sea water is VAN'T HOFF'S solution and is neutral in reaction, thus according with the reaction of all other solutions employed.

The superiority of the sea water solutions whose concentration does not exceed 0.35 *m* can be easily seen from table V. It is plain that the artificial sea water and the natural sea water of the same concentration are by far the most superior of any salt mixtures tried, and though in the last table it is compared with the most favorable salt mixtures and those which appear to approach most closely to a balanced solution, it excels even those to quite a marked extent. RINGER'S solution, not having the magnesium salt which seems to act so beneficially in balancing solutions for *B. subtilis*, gives only a fair ammonification coefficient as compared with other salt mixtures.

SERIES VI

It seemed of interest to see what effect the addition of magnesium to the RINGER'S solution would have, and a series was therefore arranged to show this.

TABLE VI

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS
PEPTONE CONTENT 0.91 PER CENT.

Number	Culture solution	N as NH_3 formed in cultures, in mg
1	RINGER'S solution	19.74
2	RINGER'S solution + 10 $MgCl_2$	26.44
3	Artificial sea water	31.41
4	Peptone in brook water	39.89
5	Peptone in distilled water	42.51
6	100 NaCl, 10 $MgCl_2$, 10 KCl, 2 $CaCl_2$	28.42

It is quite evident that the addition of magnesium to RINGER'S solution has a very favorable effect, and by slightly changing the proportion of the constituents we could easily obtain what appears

here to be the optimal solution. The brook water solution was inserted here to add another solution to the number of those which allow so vigorous an ammonification owing to the low osmotic pressure. The artificial sea water, however, still remains far superior to any other salt mixture thus far tried.

SERIES VII

In order to compare to the best advantage the various salt mixtures with the artificial sea water and distilled water solutions, it was decided to arrange in one series the best representatives of the single, binary, ternary, and quaternary salt solutions, along with the artificial sea water and the distilled water peptone solutions.

TABLE VII

ALL QUANTITIES GIVEN REFER TO CUBIC CENTIMETERS OF 0.35 *m* SOLUTIONS
PEPTONE CONTENT 0.91 PER CENT.

Number	Culture solution	N as NH_3 formed in cultures, in mg
1	100 NaCl	20.10
2	{ 100 NaCl, 10 $MgCl_2$ }	23.39
3	{ 100 NaCl, 10 $MgCl_2$, 5 $CaCl_2$ }	25.88
4	{ 100 NaCl, 10 $MgCl_2$, 10 KCl, 2 $CaCl_2$ }	28.42
5	Artificial sea water	31.41
6	Peptone in distilled water	42.51

We see here to good advantage the beneficial effects of balancing solutions. Of the large variety of single salts and combinations of salts of various kinds, there was none that could quite approach in efficiency the artificial sea water or the natural sea water of the same concentration, and we are obliged to accept the fact that for bacteria (or at least for that group represented by *B. subtilis*) physiologically balanced solutions are necessary if optimal development of the bacteria is sought for.

DISCUSSION OF RESULTS

The number of combinations of salts that could be experimented with in a manner similar to that above given is of course unlimited,

and it is impossible to make determinations of all of them, but in the foregoing series of experiments enough combinations have been tried and sufficient evidence has been adduced to make it quite clear that, for at least one class of bacteria, sea water is a physiologically balanced solution just as truly as it is for animals and the higher plants. Therefore, although some of the results exhibit some puzzling aspects, the prime object of these investigations—to discover whether sea water or other balanced solutions are superior to unbalanced solutions—is attained. The inexplicable facts which are bound to arise in such investigations must be accounted for by future research, but the universality of LOEB'S conception of "physiologically balanced solutions" is more firmly grounded than ever. To the experimental proof of it for animals in the ingenious experiments of LOEB himself are added the no less remarkable results of OSTERHOUT[5] in his work on marine as well as freshwater and terrestrial plants, and to both of these are now added the results above described on the third great class of living organisms which seem to hold the same general relation to balanced solutions as the other two.

SUMMARY

1. The ammonifying power of *B. subtilis* is stronger in artificial sea water or in natural sea water of the same concentration than in any other salt mixture.

2. Sea water may be looked upon therefore as a physiologically balanced solution for *B. subtilis* as truly as it is for animals and the higher plants.

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[5] BOT. GAZETTE **42**:127–134. 1906; **44**:259–272. 1907.